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ORIGINAL PAPER

Low temperature, IBA concentrations and optimal time for adventitious rooting of *Eucalyptus benthamii* mini-cuttings

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Abstract: *Eucalyptus benthamii* is a forest species of economic interest that has difficulty with seed production and also is considered to have difficulty with adventitious rooting using propagation techniques, such as cutting or mini-cutting. We aimed to assess the adventitious rooting percentage under different storage times in low temperatures and at various IBA (indole-3-butyric acid) concentrations to determine the optimal time of permanence for rooting *Eucalyptus benthamii* mini-cuttings in a greenhouse. Shoots collected from mini-stumps cultivated in a semi-hydroponic system were used to obtain the mini-cuttings. For the first experiment, the mini-cuttings were stored at 4°C for 0 (immediate planting), 24, 48, 72, 96 and 120 h. The second experiment evaluated the rooting dynamic to determine the optimal time of permanence for mini-cuttings in a greenhouse. The basal region of the mini-cutting was treated with various IBA solutions: 0 (free of IBA), 1,000, 2,000, 3,000 and 4,000 mg·L^{-1}. Every seven days (0 (immediate planting), 7, 14, 21 and 28 days), destructive sampling of the mini-cuttings was performed to evaluate the histology of the adventitious rooting. *Eucalyptus benthamii* mini-cuttings should be rooted immediately after the collection of the shoots. The 2,000 mg·L^{-1} IBA concentration induced a greater speed and percentage of adventitious rooting, and an interval of 35 to 42 days was indicated for permanence of the mini-cuttings in the greenhouse. Exposure to low temperature induced adventitious root formation with diffuse vascular connections.

Keywords: rhizogenesis, plant cloning, mini-cutting technique, histological analysis, indole-3-butyric acid.

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Introduction

Eucalyptus benthamii, known as "Camden White Gum," grows naturally on the east coast of New South Wales, Australia, at approximately 34°00' S latitude and 150°30' E longitude. The species is mainly confined to the southwest of Sydney in the floodplains along the Nepean River and its tributaries (Benson 1985; Benson and McDougall 1998). In keeping with the adaptive conditions of the area, the *Eucalyptus benthamii* can tolerate low temperatures, is tolerant to frost (Jovanovic and Booth 2002) and survives in absolute minimum temperatures from -6°C to -10°C (Lin et al. 2003). This species is confined to a small population of 6,500 trees in the Kedumba Valley and three remaining populations along the Nepean River until the Bents Basin, Wallacia, and in Camden, where it is considered vulnerable to extinction in its naturally occurring habitat (Butcher et al. 2005).

Recently used in commercial forest plantations, the species has exhibited limited seed production, and when it is available, the prices are high. Vegetative propagation uses many alternative techniques to clone genotypes, including pre-selected matrices for each region, considering the tolerance to cold, drought, pests

and diseases, among other characters of interest. However, the low rate of adventitious rooting limits the application of these biotechnology techniques to foster the growth of *Eucalyptus benthamii* in commercial plantations and limits its progress in breeding programs. Currently, there have not been many advances in the breeding programs of this species, although the mini-cutting technique provides excellent results for the production of cloned material from selected trees considered difficult to root, including hybrid and transgenic plants (Wendling et al. 2000; Brondani et al. 2010; Wendling et al. 2010).

The application of exogenous plant growth regulators, as the auxins group, can increase the success of adventitious rooting in vegetative propagules collected from selected genotypes of *Eucalyptus* and the indole-3-butyric acid (IBA) is the most used (Wendling et al. 2000; Wendling and Xavier 2005; Almeida et al. 2007; Schwambach et al. 2008; Brondani et al. 2010; Wendling et al. 2010). However, IBA concentrations have not been established for the adventitious rooting of *Eucalyptus benthamii* mini-cuttings. Despite all the advantages of vegetative propagation (Hartmann et al. 2011), the difficulty in inducing adventitious rooting from mature trees, the variation between clones, the sensitivity of mini-cuttings to environmental conditions, the need for faster collection of shoots, the storage of vegetative propagules from the clonal mini-garden at low temperatures, the management of cuttings in a greenhouse and the need for a better synchronized production schedule are problems that the forest sector aims to overcome using biotechnological techniques (Ferreira et al. 2004; Goulart and Xavier 2008).

Even with all the advances in mini-cutting techniques, there are few scientific reports about the effectiveness of this technique in optimizing nursery installations for the production of *Eucalyptus* clones from genotypes that are considered difficult to root (e.g., subtropical *Eucalyptus*). Based on this fact and considering the favorable conditions for the development of disease during the induction of adventitious roots in a greenhouse, related studies on the rooting dynamics of mini-cuttings and its organogenic origin are important.

We aimed to evaluate the cloning of *Eucalyptus benthamii* through the mini-cutting technique for the following purposes: (1) to determine the best IBA concentration for the induction of adventitious roots, (2) to determine the induction of adventitious rooting in mini-cuttings submitted at different storage times in low temperatures, (3) to determine the optimum time of permanence for mini-cuttings rooted in a greenhouse. In this study, we also aimed to discuss some possible problems in the complex process of adventitious root induction in mini-cuttings and to perform a histological study of the origins of vascular connections.

Material and methods

Source of plant material and management

Shoots collected from mini-stumps of selected genotypes of *Eucalyptus benthamii* Maiden & Cambage (BP101, BP118 and BP120 clones) were used as the source of vegetative propagules. The mini-stumps were planted in a 10 × 10 cm clonal mini-garden in a semi-hydroponic system. The mini-stump consisted of a plant with 7 cm of length and containing shoots available for collection at 90 days (Fig. 1A-B). The mini-stumps were propagated by conventional cutting methods (Stape et al. 2001; Schwambach et al. 2008; Wendling et al. 2010) from trees at two years and six months of age. The nutrient solution was composed of macro- and micronutrients, which were distributed at a flow of 5 $L \cdot m^{-2}$ per day (Table 1).

Fig. 1 Details of the clonal mini-garden of *Eucalyptus benthamii* cultivated in full-sun conditions in a semi-hydroponic system with sand. (A) Planting of the clones; (B) mini-stumps containing shoots available for collection at 90 days after installation. Yellow arrow indicates a mini-stump. Barr = 10.0 cm.

Preparation of mini-cuttings

Shoots from the mini-stumps were gathered in the 5th collection of the shoots (≈ 150 days after installation) for the preparation of the mini-cuttings. The shoots were 6±1 cm long and had two upper pairs of buds with the leaves reduced by 50% and two

lower pairs without leaves. The shoot apex (i.e., shoot tip) was left (Fig. 2A-B).

Table 1. Composition of nutritive solution for the fertigation of a clonal mini-garden of *Eucalyptus benthamii*.

Nutrient		Nutritive solution* ($mg \cdot L^{-1}$)
$N\text{-}NO_3^-$		90.11
$N\text{-}NH_4^+$		61.06
P		29.62
Ca		128.99
K		209.76
S		107.15
Mg		36.99
B		0.481
Cu		0.061
Fe		5.000
Mo		0.020
Mn		1.463
Zn		0.065
Source of macro- and micronutrient	MF / MW	($mg.L^{-1}$)
Calcium nitrate (Labsynth®)	$Ca(NO_3)_2.4H_2O$ / 236.15	760.0
Ammonium sulfate (Merck®)	$(NH_4)_2SO_4$ / 132.14	225.0
Monoammonium phosphate (Mallinckrodt®)	$NH_4H_2PO_4$ / 115.03	110.0
Potassium chloride (Ecibra®)	KCl / 74.56	400.0
Magnesium sulfate (Mallinckrodt®)	$MgSO_4.7H_2O$ / 246.48	375.0
Manganese sulfate (Ecibra®)	$MnSO_4.H_2O$ / 169.01	4.500
Boric acid (Ecibra®)	H_3BO_3 / 61.83	2.750
Zinc sulfate (Mallinckrodt®)	$ZnSO_4.7H_2O$ / 287.54	0.285
Copper sulfate (Mallinckrodt®)	$CuSO_4.5H_2O$ / 249.68	0.240
Iron sulfate (Synth®)	$FeSO_4.7H_2O$ / 278.02	24.89
Sodium - EDTA (Nuclear®)	Na_2-EDTA.$2H_2O$ / 372.24	33.40
Sodium molybdate (Merck®)	$Na_2MoO_4.2H_2O$ / 241.95	0.050

* The pH was adjusted to 6.2 at 25°C with hydrochloric acid (HCl) or sodium hydroxide (NaOH), both at 1 M. MF = molecular formula, MW = molecular weight.

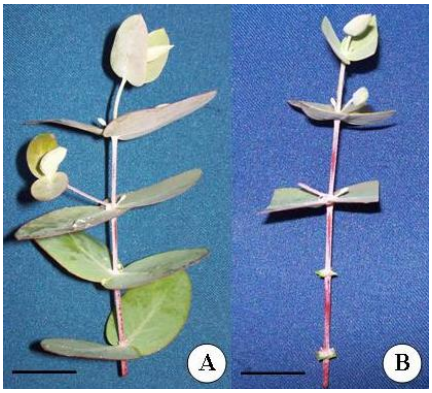

Fig. 2 Details of the preparation of *Eucalyptus benthamii* mini-cuttings. (A) Shoot containing four pairs of buds and the shoot tip; (B) mini-cuttings standardized. Barr = 1.5 cm.

Storage time in low temperature

Mini-cuttings of BP101, BP118 and BP120 clones were exposed to 4°C in a cold chamber for 0 (control - immediate planting), 24, 48, 72, 96 and 120 h. To induce adventitious rooting, the basal region of the mini-cutting was dipped for 10 s in a water-alcohol solution (1:1, water:alcohol, v/v) containing 2,000 $mg \cdot L^{-1}$ IBA. The survival percentage was evaluated in a greenhouse (35 days), shade house (14 days) and an area of full-sun (30 days). In the area of full-sun, the total length of the root system (TLRS), root volume (RV) and adventitious rooting percentage (AR) of the mini-cuttings were measured. Histological analysis of the adventitious rooting was performed to verify the quality of the vascular connection. The experiment was conducted in a completely randomized design with a split plot in time using three clones and six periods of cold exposure with five replications of ten mini-cuttings per replication.

Effect of IBA on adventitious rooting

The basal region of the mini-cutting (Fig. 2B) was immersed for 10 s in a water-alcohol solution (1:1, water:alcohol, v/v) in the following concentrations: 0 (control - no IBA), 1,000, 2,000, 3,000 and 4,000 $mg \cdot L^{-1}$ IBA. The mini-cuttings were maintained in a greenhouse to induce rhizogenesis. The adventitious rooting percentage (AR) (Ferreira et al. 2004) and the length of the longest root (LLR) were measured. Nine samples of the basal region of the mini-cutting were collected at 0 (immediate planting), 7, 14, 21 and 28 days after treatment with IBA. The experiment was conducted in a completely randomized design with a split plot in time using three clones, five IBA concentrations and seven evaluation times with five replications of fifty mini-cuttings per replication.

Histological analysis

The samples were fixed in formaldehyde and glutaraldehyde solution (Karnovsky 1965) and subjected to three vacuum series (≈ 620 $kgf \cdot cm^{-2}$) of 15 minutes each to remove the air. Next, the samples were dehydrated using an ethyl-alcohol series in increasing concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%, v/v), remaining in each solution for 10 minutes, and then they were embedded in a hydroxyethyl methacrylate resin (Leica®). The blocks containing the samples were sectioned longitudinally or transversely to a 5 µm thickness using a manual rotary microtome coupled with a type C razor. The sections were stained with toluidine blue (0.05%, v/v) in a phosphate buffer and citric acid (Sakai 1973) and mounted on slides with a synthetic resin (Entellan®). The histological slides were analyzed and photomicrographed using a light microscope (Zeiss-Jenemed2®) in micrometer scale, and the images were captured using a Samsung camera (SDC-313). Ten samples were used for each replication.

Conditions of adventitious rooting

The mini-cuttings were inserted at a 2 cm depth in a substrate composed of a mixture of medium vermiculite and an organic substrate base of decomposed pine bark (Basaplant®) (2:1, v/v). Fertilization was not performed. Small plastic conical tubes (55 cm^3) were used as the cultivation container. The tubes had been previously disinfected with a solution containing sodium hypochlorite (NaOCl) at 0.25% active chlorine (v/v) for 48 h. The automated greenhouse maintained an air temperature between 25°C and 31°C and an air relative humidity greater than 80%. The shade house was covered with 50% black mesh. The microsprinkler system of the shade house and the full-sun area applied a water system pressure of 2.0 $kgf \cdot cm^{-2}$ at predetermined intervals controlled by a timer.

Statistical analysis

The data were submitted to a Hartley test ($p<0.05$) to verify the homogeneity of variance between the treatments and to a variance analysis (ANOVA) ($p<0.01$ and $p<0.05$). According to the significance of the ANOVA, the data of the qualitative factors were compared by Tukey test ($p<0.05$), and the data of the quantitative factors were submitted to polynomial or logistical regression analysis ($p<0.05$).

Results

Storage time in low temperature

The time of cold exposure had little influence on the mini-cuttings survival in the greenhouse even when the mini-cuttings were subjected to 4°C for 120 h ($p < 0.01$ and $p < 0.05$). During the acclimation phase in the shade house, we observed similar results, but the treatments with 24 and 48 h of exposure to cold had the lowest survival rate ($p < 0.01$ and $p < 0.05$). The other treatments of exposure to cold resulted in more than 87% survival, which is considered high for the species studied. The overall average percentages of survival for the mini-cuttings in the greenhouse and shade house were 97.8% and 88.7%, respectively. There was no significant effect of the time of exposure to low temperature on the mini-cuttings survival in the greenhouse ($p < 0.01$ and $p < 0.05$) and during acclimation in the shade house ($p < 0.01$ and $p < 0.05$), independent of the evaluated clone.

The biggest difference of rooting percentage between the clones in relation to the time of cold exposure occurred in the area of full-sun ($p < 0.05$). The BP101 (Fig. 3A) clone showed an increase in the rooting percentage until 48 hours of exposure to cold. After 48 h, there was a reduction in the rooting percentage in relation to the control, reaching zero percent when the storage time was 120 h. The BP118 (Fig. 3B) and BP120 (Fig. 3C) clones showed linear decreases with increasing time of low temperature exposure, and the reduction in rooting was 15% to 20%, respectively, compared to the control (planted immediately after collection of the shoots).

Clone BP118 showed the highest values for the total length of the root system (Fig. 3D) and the root volume (Fig. 3E), differing significantly from the other genetic materials ($p < 0.01$). This result demonstrates that the genetic materials differed in relation to the growth and development of the adventitious root and that proper management in the nursery should be specific to each genetic material to produce clones of *Eucalyptus benthamii* in an appropriate quality and quantity. The root volume (Fig. 3F) decreased linearly with an increasing time of cold exposure regardless of the genetic material evaluated ($p < 0.05$). The reduction in root volume reached 50% when stored at low temperature for 120 h compared to time 0 (planted immediately after collection of the shoots), impairing the quality of the adventitious root induced.

Histological analysis of the mini-cuttings exposure to cold for 72 h revealed normal vascular connections of the adventitious root directly with the cambium (Fig. 4A) regardless of the clone. Following longer periods of exposure to cold (96 and 120 h), the vascular connection of the adventitious root with the vascular cambium became diffuse, i.e., showing set of cells in disarray (Fig. 4B). With these results, it was evident that prolonged exposure to low temperature affected the organogenic capacity of the plant tissue and the quality of the adventitious root.

Effect of IBA on adventitious rooting

The treatment without IBA (Fig. 5A) showed root induction only at 28 days (22.2% rooting) ($p<0.05$), similar to the concentration of 1,000 $mg \cdot L^{-1}$ IBA (33.3% rooting) (Fig. 5B). The concentrations of 2,000 $mg \cdot L^{-1}$ (Fig. 5C), 3,000 $mg \cdot L^{-1}$ (Fig. 5D) and 4,000 $mg \cdot L^{-1}$ IBA (Fig. 5E) showed rooted mini-cuttings in 21 days and at 28 days, with 44.4%, 44.4% and 55.5% rooting, respectively ($p<0.05$). This result suggests the importance of IBA application for achieving greater adventitious rooting speed and induction in concentrations equal to or greater than 2,000 $mg \cdot L^{-1}$ IBA (Fig. 5C). At 45 days, the rooting percentage showed similar values in relation to the IBA concentration (60.0% to 77.7% rooting) independent of the clone evaluated

The treatments without IBA (Fig. 6A) and with 1,000 $mg \cdot L^{-1}$ IBA (Fig. 6B) showed slower growth of the root in relation to the other treatments ($p<0.01$), with 1.3 and 1.1 cm length at 28 days, respectively. The highest values of the length of the longest root were obtained with the treatments of 2,000 $mg \cdot L^{-1}$ (Fig. 6C), 3,000 $mg \cdot L^{-1}$ (Fig. 6D) and 4,000 $mg \cdot L^{-1}$ IBA (Fig. 6E), which showed roots with 1.6, 2.8 and 4.0 cm length, respectively, at 28 days ($p<0.01$). At 35 days, all the treatments except the control (without IBA) presented values for the LLR greater than 5 cm. At 42 days, the treatment without application of IBA showed the lowest value for the LLR. The LLR for the concentrations of 1,000 $mg \cdot L^{-1}$ (Fig. 6B) and 2,000 $mg \cdot L^{-1}$ (Fig. 6C) showed similar values between 35 and 42 days.

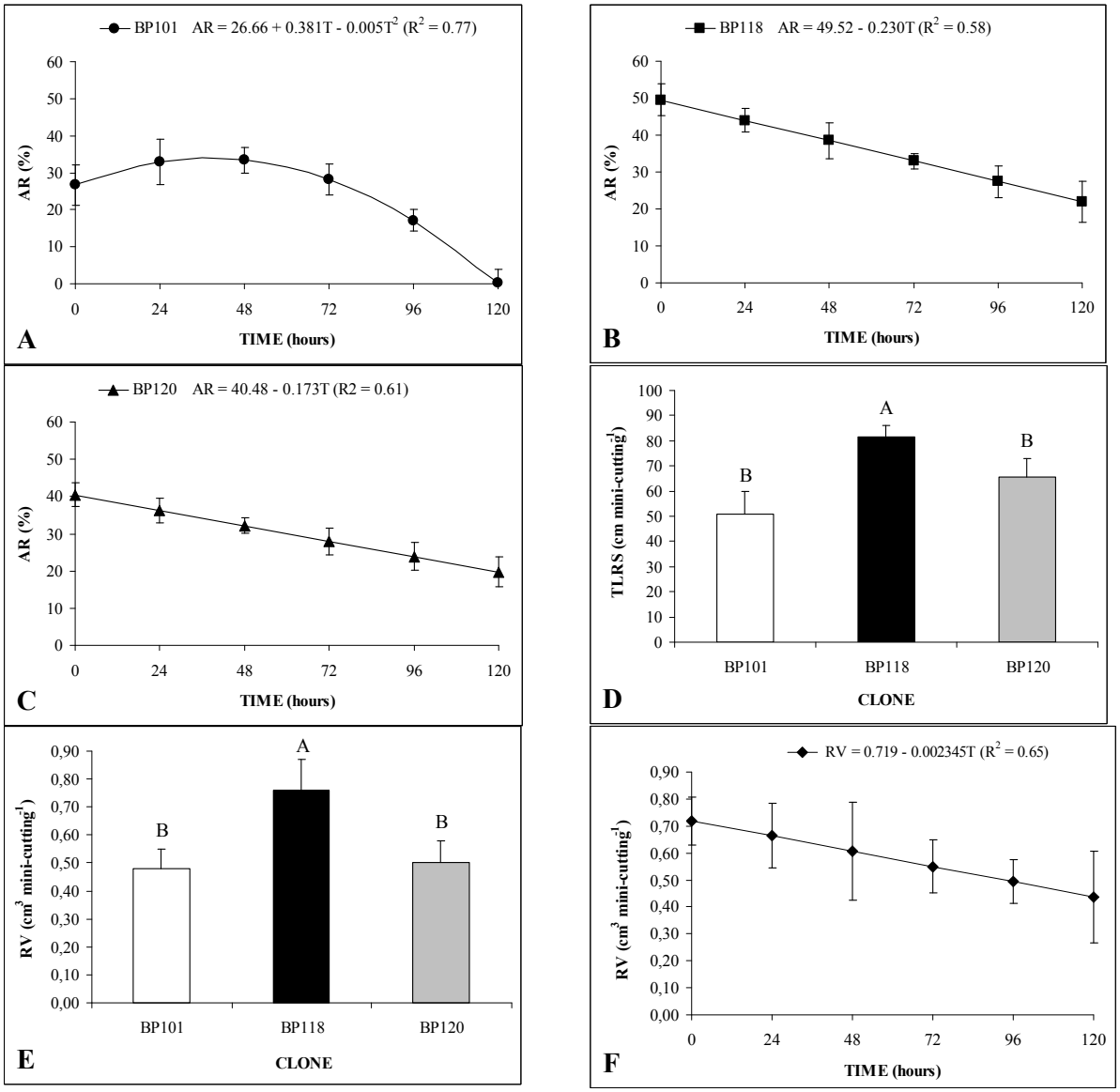

Fig. 3 Adventitious rooting of *Eucalyptus benthamii* mini-cuttings in an area of full-sun, 79 days. (A, B and C) Percentage of adventitious rooting (AR) in relation to the clone (BP101, BP118 and BP120) and the time of low temperature exposure. (D) Total length of the root system (TLRS) in relation to the clone; mean values followed by the same letter do not differ significantly by Tukey test (p<0.05). (E) Root volume (RV) in relation to the clone; mean values followed by the same letter do not differ significantly by Tukey test (p<0.05). (F) Root volume (RV) in relation to the time of low temperature exposure. The data are presented as the average ± standard deviation.

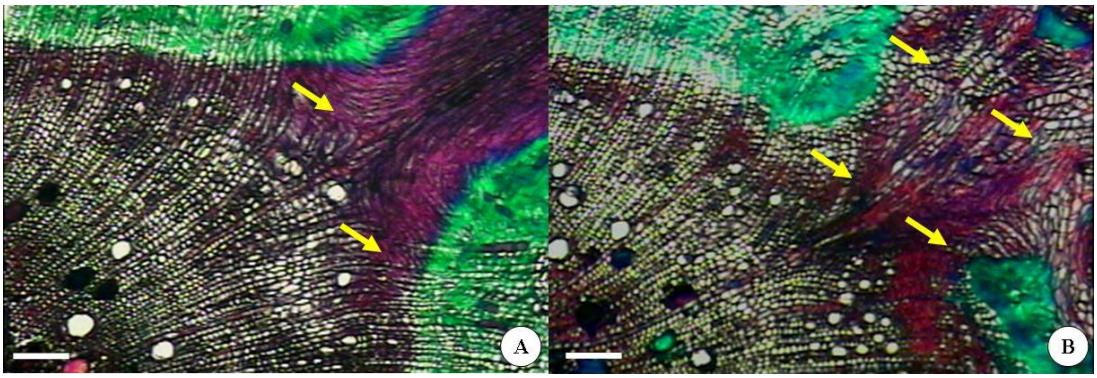

Fig. 4 Detail of the histological view of the adventitious rooting in *Eucalyptus benthamii* mini-cuttings, indicating the vascular connection with the cambium. (A) Normal connection; (B) diffuse connection. Yellow arrows indicates the vascular connection. Bar = 100 µm.

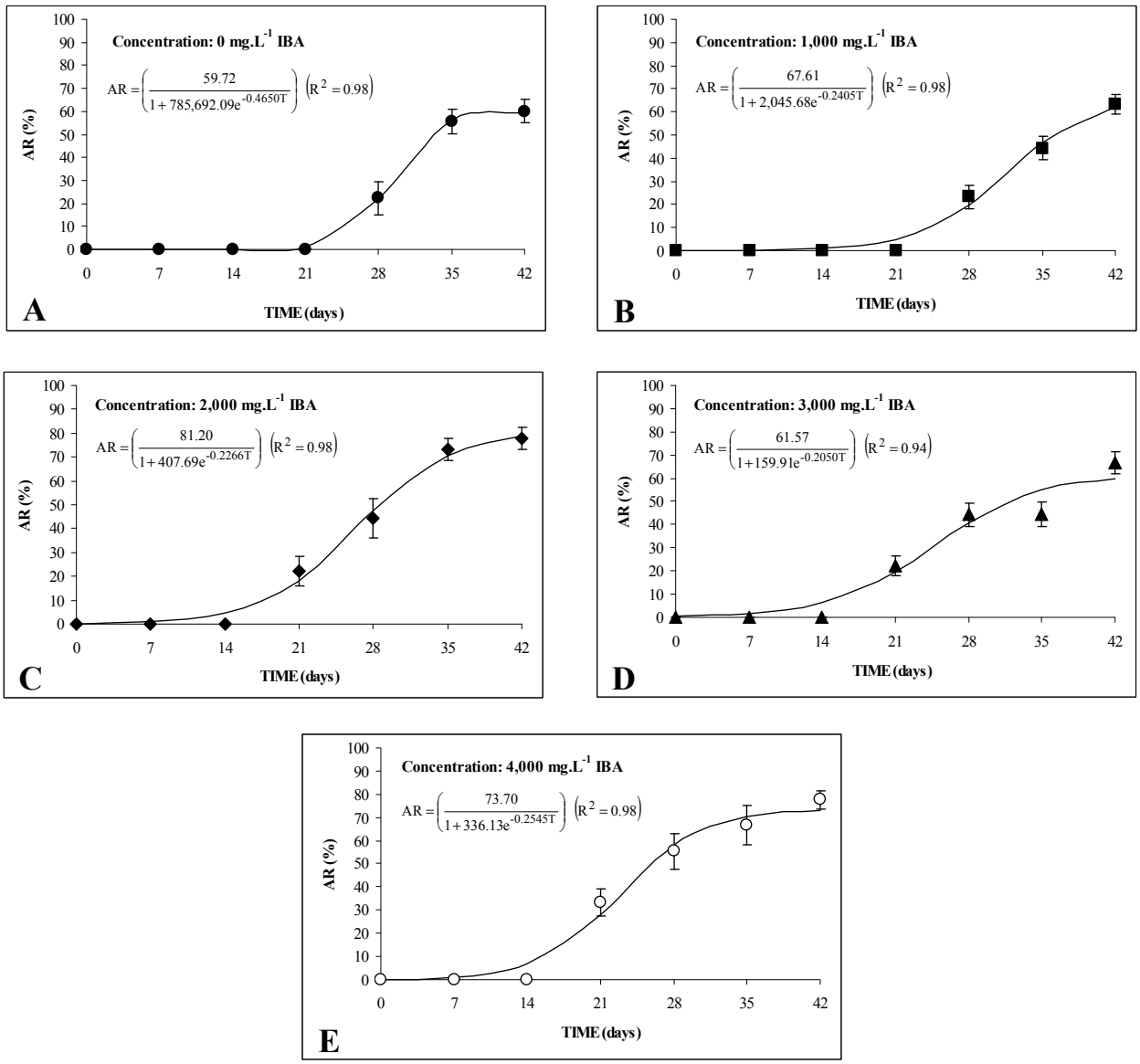

Fig. 5 Adventitious rooting percentage (AR) of *Eucalyptus benthamii* mini-cuttings in relation to the evaluation time and IBA concentration. (A) Without IBA application, (B) application of 1,000 mg·L^{-1} IBA, (C) application of 2,000 mg·L^{-1} IBA, (D) application of 3,000 mg·L^{-1} IBA, (E) application of 4,000 mg·L^{-1} IBA. The data are presented as the average ± standard deviation.

In general, the presence of IBA in the concentrations of 2,000, 3,000 and 4,000 mg·L^{-1} (Fig. 6C-E) promoted positive results for all the clones. The concentration of 2,000 mg·L^{-1} IBA (Fig. 6C) resulted in better values for the rooting speed and the length of the longest root. The treatments of 3,000 mg·L^{-1} and 4,000 mg·L^{-1} IBA (Fig. 6D-E) also showed promising effects for adventitious rooting, but there is no justification for using these concentrations for these clones (BP101, BP118 and BP120) because the responses were similar to the concentration of 2,000 mg·L^{-1} (Fig. 6C).

The histological analysis of adventitious rooting in the beginning of the experiment (0 days) showed a typical arrangement of the cortex, vascular cambium and conductive vessels and showed the presence of subepidermal duct conductors (Fig. 7A-E). At 7 days, there was no change in the cellular organization of the tissues (Fig. 7F-J), but at 14 days, meristematic centers formed near the vascular cambium in the treatments with 0, 1,000 and 2,000 mg·L^{-1} IBA (Fig. 7K-M) and the treatments with 3,000 and 4,000 mg·L^{-1} IBA showed destruction of the tissues (Fig. 7N-O). At 21 days, there was an apex radial polarization and induction of adventitious roots in the treatments with 0, 1,000, 2,000 and 3,000 mg·L^{-1} IBA (Fig. 7P-S), but there was a disruption of the tissue at a concentration of 4,000 mg·L^{-1} IBA (Fig. 7T). At 28 days, there was induction of adventitious roots with a connection to the vascular cambium for the treatments with 0, 1,000, 2,000 and 3,000 mg·L^{-1} IBA (Fig. 7U-Z). However, there was induction of an external callus and total disruption of the tissue in the treatment with 4,000 mg·L^{-1} IBA (Fig. 7W) with subsequent

adventitious rooting.

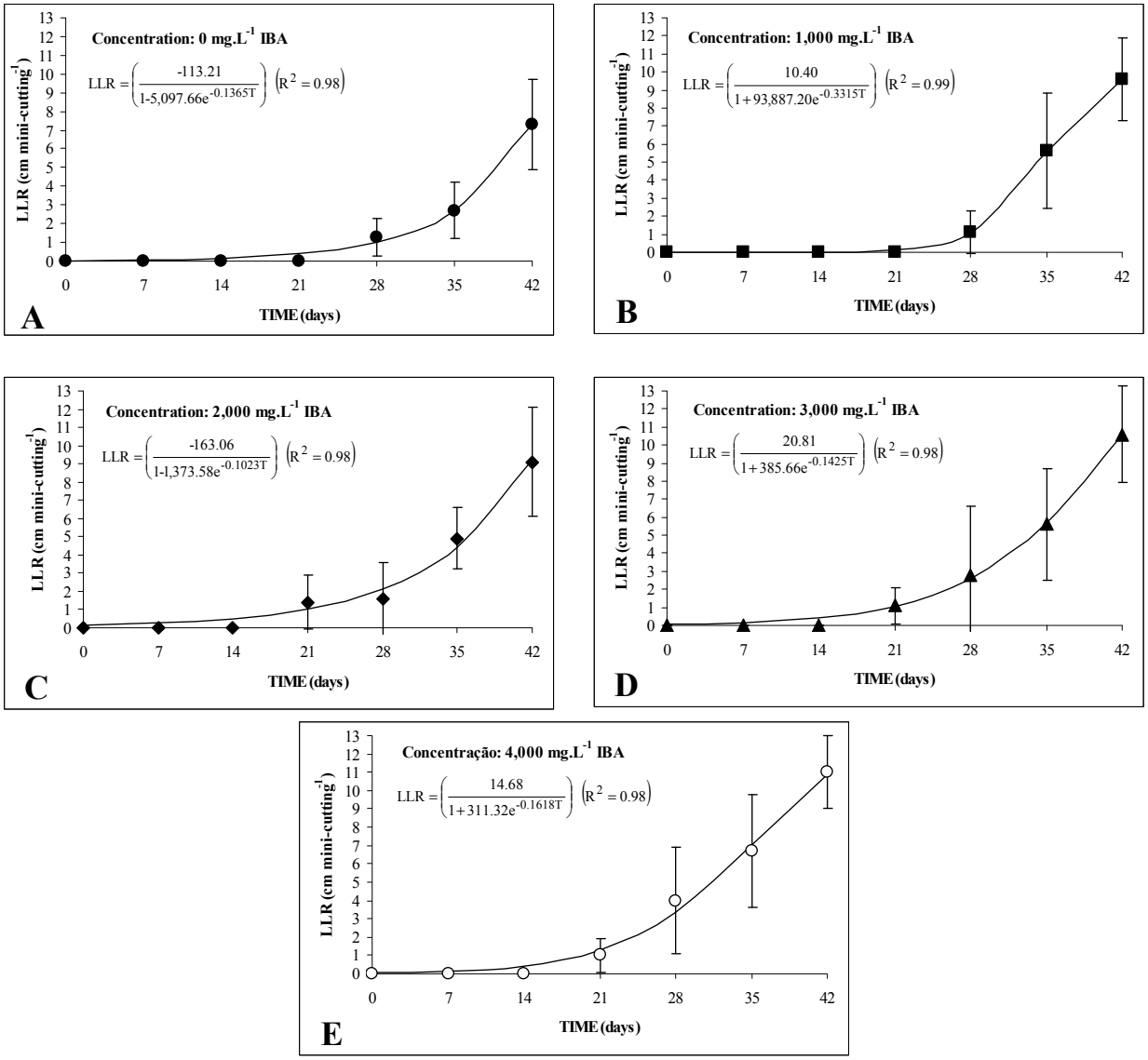

Fig. 6 Length of the longest root (LLR) of *Eucalyptus benthamii* mini-cuttings in relation to the evaluation time and IBA concentration. (A) Without IBA application, (B) application of 1,000 mg·L^{-1} IBA, (C) application of 2,000 mg·L^{-1} IBA, (D) application of 3,000 mg·L^{-1} IBA, (E) application of 4,000 mg·L^{-1} IBA. The data are presented as the average ± standard deviation.

Discussion

Numerous protocols involving cutting and mini-cutting techniques were developed for *Eucalyptus* species, but no suitable protocol of vegetative propagation was worked for *E. benthamii*, necessitating adjustments to increase the quality and quantity of clonal production. In terms of mini-cutting technique, studies demonstrated the viability of shoots production for adventitious rooting in a greenhouse to ensure a constant production of clones throughout the year (Cunha et al. 2005), but survival rates and mini-cutting rooting were not measured. Even with advances in biotechnology, little is known about the organogenic characteristics of adventitious roots or about the speed of rooting induction. These parameters could be used to mathematically model the permanence time of mini-cuttings in a greenhouse (Ferreira et al. 2004; Goulart and Xavier 2008) and to optimize clonal production in the nursery.

The application of auxin in the basal region of cuttings and mini-cuttings has been used worldwide to promote the adventitious rooting of woody species (Hartmann et al. 2011; Hunt et al. 2011; Schwambach et al. 2008; Wendling et al. 2010) and to influence the micro-cuttings survival and the rooting of species considered recalcitrant to rooting (Bennett et al. 2003; Schwambach et al. 2005). In the cases of auxin application, great variation was observed among the concentrations, formulations and forms of application of plant growth regulator (Wendling et al. 2000; Fogaça and Fett-Neto 2005; Wendling and Xavier 2005; Almeida et al. 2007; Schwambach et al. 2008; Wendling et al.

2010) as well as other factors considered intrinsic based on the genetics (Stape et al. 2001; Bennett et al. 2003; Corrêa and Fett-Neto 2004). It is known that IBA enhances the induction of adventitious roots in vegetative propagules, but depending on the management style and the genetic material, the concentrations should be adjusted to obtain better rooting rates (Corrêa and Fett-Neto 2004). In this study, the *E. benthamii* responded positively to rooting in concentrations equal to or greater than 2,000 mg·L^{-1} IBA, as reported for *E. cloeziana* (Almeida et al. 2007) and *E. benthamii* x *E. dunnii* (Brondani et al. 2010).

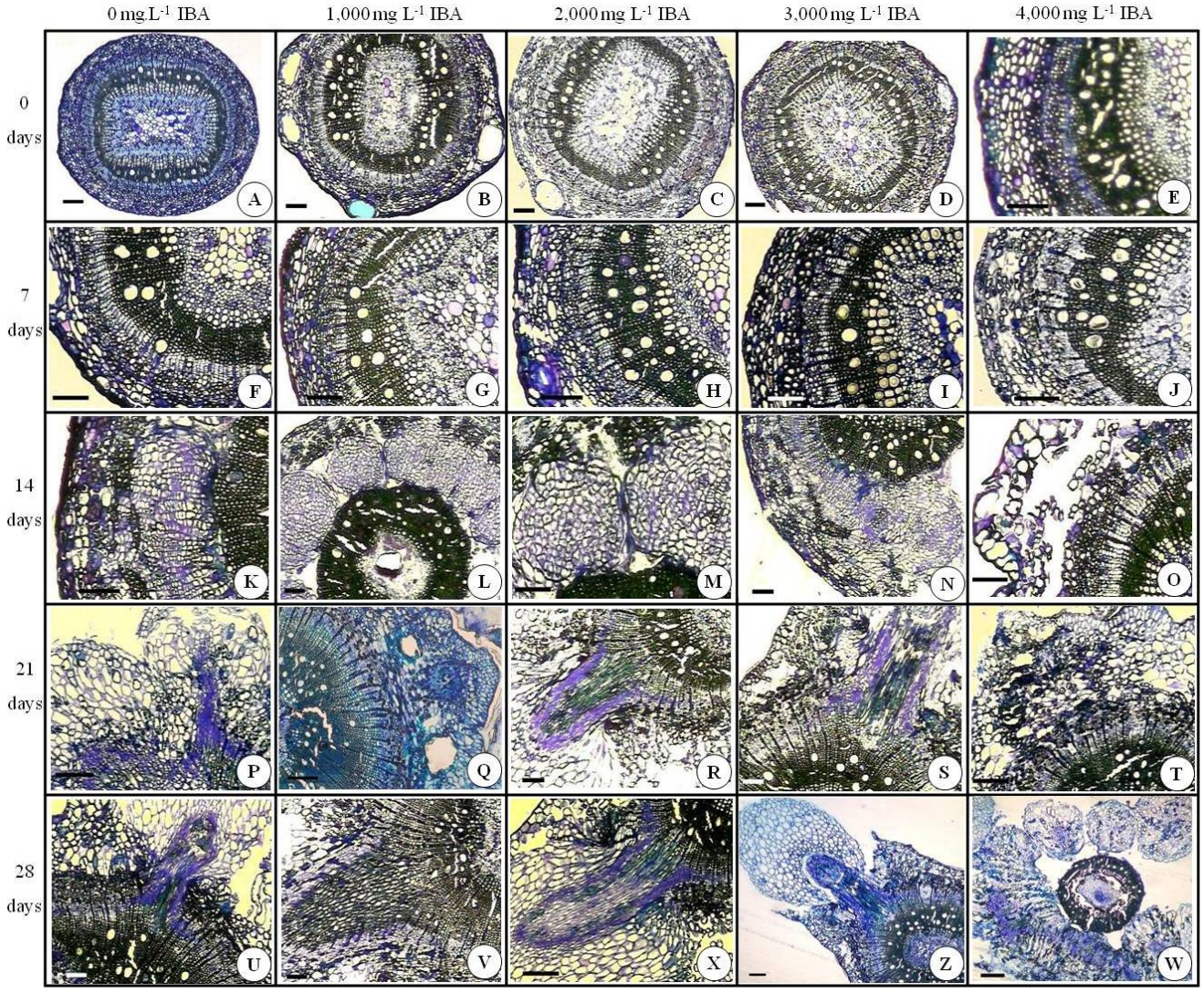

Fig. 7 Histology of the induction and development of adventitious rooting in *Eucalyptus benthamii* mini-cuttings. (A-E) Transverse section of the basal region of the mini-cutting after treatment with IBA, 0 days; (F-J) transverse section of the basal region of the stem treated with IBA, 7 days; (K-O) transverse section of the basal region of the mini-cutting treated with IBA showing the formation of meristematic centers in the cortex, 14 days; (P-T) transverse section of the basal region of the mini-cutting treated with IBA showing the induction of the adventitious root, 21 days; (U-W) transverse section of the basal region of the mini-cutting treated with IBA showing the adventitious root, 28 days. Bar: 100 μm.

As to the inductive effect of the IBA, adventitious roots may arise in a variety of tissues based on clusters of mature cells that recover their ability of cell division and develop in the root apical meristem similarly to the formation of lateral roots (Fogaça and Fett-Neto 2005). Adventitious rooting appears to be especially controlled by endogenous levels of auxins (Luckman and Menary 2002; Li et al. 2009; Zhu et al. 2010). Exogenous applications of auxins increase the rooting percentage due to their ability to act in plant tissues located near the region of contact with the plant growth regulator (Fogaça and Fett-Neto 2005; Husen and Pal 2007) and can also be related to the timing of IBA applications (Luckman and Menary 2002) and concentrations (Wendling et al. 2010). Therefore, the application of IBA increases the concentration of endogenous auxins, and the accumulation of the IBA in the basal region of the vegetative propagules acts as a metabolizing agent and signal to induce rooting (Husen 2008). During external contact with the cell, the IBA induces changes in the metabolism of enzymes, carbohydrates, RNA, DNA and proteins, and these changes in the rooting zone may inhibit or promote regeneration of adventitious roots, mainly

during cell division and differentiation (Baltierra et al. 2004; Dai et al. 2004; Husen and Pal 2007; Komatsu et al. 2011).

Low temperature may inhibit the activity of endogenous auxins (Corrêa and Fett-Neto 2004). This physiological event can occur when the collection of the shoots takes place far from the place of cutting preparation, when the vegetative propagules are transported for long periods under low temperature (usually at 4°C). Submitting the propagules of *E. benthamii* to low temperature for prolonged periods reduces their rhizogenic potential. Therefore, we observed a reduction in the cellular competence for adventitious rooting of *E. benthamii* mini-cuttings proportional to the increase in storage time of the vegetative propagules at low temperature. According to the results, we recommend the planting of *E. benthamii* mini-cuttings in the rooting substrate immediately after the collection of the shoots from the mini-stumps, considering the significant reduction in the quality of induction and root development in cuttings stored at low temperature for long periods of time.

Studies have shown a reduction in the basipetal transport of IAA when the temperature is reduced from 25°C to 4°C (Garrido et al. 2002). This effect may have influenced the reduction in the induction of adventitious roots in the mini-cuttings stored for a long time at low temperature (i.e., 4°C). However, little is known about the effects of temperature at various stages of root development at the cellular level, particularly in woody species (Corrêa and Fett-Neto 2004), and new studies are needed to identify the interfering factors and/or physiological changes.

Another problem that occurs in forest species with rooting difficulty is that a long time of permanence in a greenhouse (e.g., high air relative humidity and air temperature) to induce rhizogenesis can cause disease and mortality of the vegetative propagules (Ferreira et al. 2004; Goulart and Xavier 2008; Zhu et al. 2010). When the vegetative propagules remain for a long time at high air temperature and air relative humidity, degradation of the auxins in the basal region occurs (Rasmussen et al. 2009), and this effect may impair the induction of adventitious rooting and promote callus formation. Therefore, it is important to establish an optimal time of permanence in a greenhouse to optimize clone production and minimize losses. With regard to *E. benthamii*, our data suggest that the mini-cuttings should remain between 35 to 42 days in a greenhouse to induce adventitious rooting, considering variations in genetics, management conditions and seasons. However, in this case, the permanence time in the greenhouse can be reduced with the advancement of improved forest techniques and by the selection of genotypes that have better rooting capabilities, considering the sensitivity of rooting in relation to the temperature variation (Corrêa and Fett-Neto 2004).

The vascular connection of the adventitious root is another key issue that must be taken into consideration to produce quality clones. According to Li et al. (2009), the formation of adventitious roots in vegetative propagules can occur in two ways: (*i*) by direct organogenesis from differentiated cellular tissues (e.g., vascular cambium) or (*ii*) from callus tissue. Generally, the presence of callus occupying a region of vascular connection is problematic because the connection with the root is very fragile and the presence of callus can compromise the functionality of the root. Therefore, it is preferable to have a vascular connection with a direct origin from the vascular cambium, which influences the proper functionality of the root, influencing the development of plant. Hunt et al. (2011) observed that IBA uptake was greatly diminished by 4 days after severance in *Pinus elliottii* var. *elliottii* × *P. caribaea* var. *hondurensis* cuttings, possibly because extensive wound callus impeded IBA uptake, affecting the morphogenesis of the cuttings (e.g., shoots and roots induction).

Callus formation in the basal region of the vegetative propagules is directly associated with the induction of adventitious roots and is an important event for cell differentiation (Baltierra et al. 2004; Amri et al. 2010; Hunt et al. 2011). However, our data showed that the induction of roots from callus formed a very weak connection with the vascular cambium and that the root can break that connection very easily during planting in the field. This weak connection can also cause the tree to fall because the connection of the root and stem may not sustain wind pressure. We observed that the roots formed without the presence of external callus had a higher stability in the vascular connection with no disruption of the tissues formed, similar to results reported for *E. globulus* (Baltierra et al. 2004).

In conclusion, the mini-cutting of *E. benthamii* should be performed immediately after the collection of the propagules to favor the quality of the adventitious root formed. A concentration of 2,000 $mg \cdot L^{-1}$ IBA induced a higher speed and rooting percentage in the vegetative propagules, and the optimum time for permanence in a greenhouse was 35 to 42 days. An anatomical analysis of the adventitious root formation revealed a diffuse vascular connection when the mini-cuttings were stored in low temperature for a long time. However, when the mini-cuttings were inserted in the substratum immediately after the shoots were collected, the vascular connection was normal and favored the quality of clone production.

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